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Cardiovirulent Coxsackieviruses and the Decay-Accelerating Factor (CD55) Receptor

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Group B coxsackieviruses are etiologically linked with many human diseases including acute myocarditis and associated chronic dilated cardiomyopathy. Well-established CVB3 cardiovirulent strains (CVB3c$_{(s)}$) with known phenotypic differences have been used to study the pathogenesis of virus-induced heart disease. The receptor-binding characteristics of *cardiovirulent* CVB3 are not known, but may represent one mechanism accounting for differences in disease virulence. In this study, interactions between CVB3c$_{(s)}$ and the decay-accelerating factor (DAF or CD55) cell surface receptor were examined. Anti-DAF monoclonal antibodies (MAbs) blocked virus binding and infection of susceptible HeLa cells. Virus binding was significantly reduced by treatment of these cells with phosphatidylinositol phospholipase C enzyme, which rendered them DAF-deficient. CVB3c$_{(s)}$ exhibited a differential propensity for the DAF receptor, as several cardiovirulent strains interacted more strongly than others. However, virus binding and infection was always most effectively blocked by MAbs directed against the SCR 2 and 3 domains of DAF, suggesting that binding occurs at a similar site(s) on the molecule for all strains. Virus binding and internalization were associated with DAF down-regulation at the cell surface, as monitored by flow cytometry analysis. Cardiovirulent CVB3 did not interact with molecules functionally and/or structurally related to DAF, including CD35, CD46, Factor H, or C4-binding protein. Adenovirus type 2 (Ad2) does not use the DAF receptor. However, competitive binding assays between Ad2 and CVB1-6, CVB3c$_{(s)}$, anti-DAF MAbs, or DAF-reduced cells indicated that DAF is associated with Ad2 receptors on the HeLa cell membrane. In summary, this study indicates that DAF is an attachment receptor for cardiovirulent CVB3 and that DAF interaction may be important in the pathogenesis of CVB-mediated heart disease. © 1998 Academic Press

INTRODUCTION

Myocarditis is defined as heart disease in which there is inflammation of the myocardium with myocardial cell necrosis (Aretz, 1987; Aretz *et al.,* 1987). It is the most common cause of acquired heart failure in children and is regarded as an important predisposing condition of dilated cardiomyopathy (DCM) in adults (Liu *et al.,* 1996; Martino *et al.,* 1994, 1995b,c; Woodruff, 1980). Yet despite its importance in both the pediatric and adult populations, there is little effective treatment available, and mortality remains high. A recent prospective clinical trial indicated that 56% of patients die within 4.3 years of diagnosis (Mason *et al.,* 1995). Coxsackievirus group B (CVB) is the most commonly identified pathogen in patients with myocarditis and DCM, having been implicated in more than 50% of cases with infectious etiology (Liu *et al.,* 1996; Martino *et al.,* 1994, 1995b,c; Woodruff, 1980).

The initial event in these disease processes is the binding of virus to its receptor(s) on host cells. The first CVB receptor to be identified was the decay-accelerating factor (DAF or CD55) (Bergelson *et al.,* 1995; Shafren *et al.,* 1995). DAF is a ~70-kDa glycophosphatidylinositol-anchored membrane protein with broad tissue distribution. It functions in the complement regulatory system, by preventing formation or causing dissociation of C3 convertases in both the classical and alternative system pathways (Hourcade *et al.,* 1989; Lublin and Atkinson, 1989; Nicholson-Weller and Wang, 1994).

CVB serotypes differ in their ability to bind to the DAF receptor. Monoclonal antibodies (MAb) to DAF blocked binding to and infection of HeLa cells *in vitro* by CVB 1, 3, and 5 protoserotypes (Bergelson *et al.,* 1995; Shafren *et al.,* 1995). DAF expression on transfected rodent cells promoted virus binding (Bergelson *et al.,* 1995; Shafren *et al.,* 1995). In contrast, protose-

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TABLE 1

Characteristics of CVB3c$_{(s)}$ Strains

Strain	Cardiovirulent characteristics	References	DAF-binding phenotype
CVB3-CG and CVB3-SH	Adapted by Woodruf and passaged in two laboratories giving rise to -CG and -SH strains. Cause severe histologic damage to heart in adolescent mouse models.	Chow *et al.* (1991); Gauntt *et al.* (1984); Van Houten *et al.* (1991)	Very strong
CVB3-NR	Animal passage selection for low mortality, but high capacity for inducing myocarditis in susceptible neonatal or weanling mice. Less cardiovirulent in young adult mice than CVB3-CG or -SH.	Chow *et al.* (1991); Wilson *et al.* (1969)	Weaker
CVB3-20	Natural isolate from a patient in California in the 1950s. Less cardiovirulent in young adult mice than CVB3-CG or -SH.	Chow *et al.* (1991); Tracy *et al.* (1992)	Weaker
CVB3-∅	Infectious laboratory variant related to CVB3-20. Originally regarded as noncardiovirulent, but a single base pair transition at nucleotide 234 (C-U in the 5′ nontranslated region) repairs a ribosomal binding site and restores the cardiovirulent phenotype.	Chapman *et al.* (1994); Tu *et al.* (1995)	Very strong
CVB3-N	Cardiovirulent large plaque strain derived from CVB3-VR30. Cloned, sequenced, and studied in murine models of virus persistence and chronic heart disease.	Kandolf *et al.* (1985, 1993); Klingel *et al.* (1992); Klump *et al.* (1990)	Weaker
CVB3-CGP1V	Generated by passaging CVB3-CG once through Vero cells to examine phenotypic / genotypic changes from virus passage through cell lines.		Very strong
CVB3-VR30	Obtained directly from ATCC and passaged once through HeLa cells to acquire stock virus. Isolated from the stool of a febrile patient with minor illness, in Connecticut in 1949. Presumably noncardiovirulent.		Additional receptor(s) needed for efficient binding to the cell surface

rotypes CVB 2, 4, and 6 did not bind to DAF (Bergelson *et al.,* 1995; Shafren *et al.,* 1995).

Virus strains which occur within each CVB serotype differ in their ability to bind to the DAF receptor. One prototype CVB3-VR30 strain was found to bind to transfected cells expressing the DAF molecule (Shafren *et al.,* 1995), whereas a second CVB3-VR30 strain did not bind to DAF on transfected cells (Bergelson *et al.,* 1995). Serial passage of the non-DAF-binding CVB3-VR30 strain through human rhabdomyosarcoma cells selected a strain (CVB3-RD; Reagan *et al.,* 1984) that was capable of binding to DAF on transfected cells (Bergelson *et al.,* 1995). Moreover, CVB strains isolated from infants with aseptic meningitis have been shown to differ in their ability to bind to DAF (Bergelson *et al.,* 1997b). Some clinical isolates of CVB 1, 3, and 5 were inhibited from infecting HeLa cell monolayers by an anti-DAF MAb (MAb IF7), whereas several other strains did not appear to interact with DAF (Bergelson *et al.,* 1997b).

CVB3 cardiovirulent strains (CVB3c$_{(s)}$) are used in a number of laboratories to study viral heart disease. These strains differ in cardiopathogenicity in several murine model systems (Chow *et al.,* 1991). The characteristics of the CVB3 strains used in this study are listed in Table 1. The capacity of each of these strains for binding receptors and infecting cells is expected to be an important part of the disease process. However, it is not known if receptor molecules such as DAF play a role in the binding and infection of host cells by *cardiovirulent* CVB3. In this study, we examined the interactions of CVB3c$_{(s)}$ with the DAF receptor molecule. It has previously been reported that prototype CVB3 and Ad2 fiber compete for receptor binding (Lonberg-Holm *et al.,* 1976). In this study, we also examined the ability of CVB3c$_{(s)}$ to compete with Ad2 binding and the ability of Ad2 to bind to DAF.

RESULTS

MAb inhibition of [^{35}S]CVB3c$_{(s)}$ binding to HeLa cells

We first examined whether anti-DAF MAb inhibits cardiovirulent CVB3 virus binding to susceptible cells (Fig. 1). Pretreatment with MAb 914 to DAF blocked the binding of all cardiovirulent [^{35}S]CVB3 strains to HeLa cells. However, the degree of binding inhibition differed for each strain. Preincubation with MAb 914 strongly reduced the binding of radiolabeled CVB3-CG, CVB3-CGP1V, CVB3-SH, CVB3-∅, and CVB3-VR30 to the cell surface, moderately reduced binding of CVB3-N and CVB3-NR, and only weakly reduced binding of CVB3-20 to HeLa cells. In contrast, there was no reduction of [^{35}S]CVB3-CG binding when cells were pretreated with control MAb 653 (anti-CD4) (inhibition, 0%; results not shown).

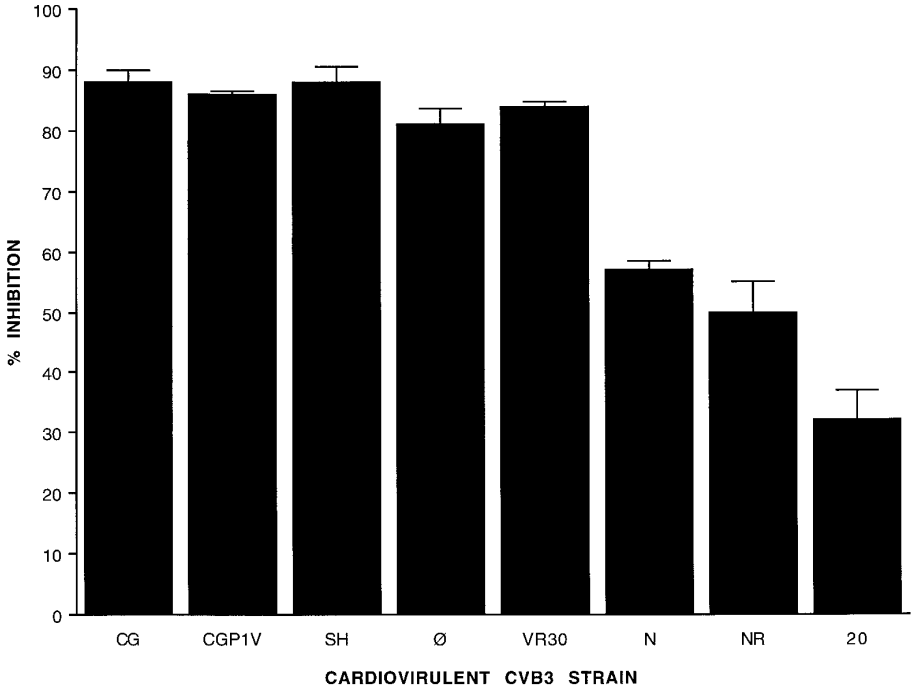

FIG. 1. Inhibition of [^{35}S]CVB3c$_{(s)}$ binding to HeLa cells by anti-DAF MAb 914. Versene-dispersed HeLa cells washed and aliquoted at 5×10^6 cells/test were resuspended in 200 μl media containing 50 μg/ml of anti-DAF MAb 914, or 200 μl media only, at 37°C for 1 h. After washing, 10^3–10^4 cpm of [^{35}S]CVB3c$_{(s)}$ was added to the cells for 1 h at 37°C. Cells were centrifuged at 1000 *g* for 3 min and the supernatant fluid and two subsequent washes were collected, as was the cell pellet. Unbound virus and cell bound virus were determined by monitoring the ^{35}S cpm in supernatants and cell pellets, by scintillation spectroscopy. Experiments were performed in triplicate for each virus strain. Inhibition of [^{35}S]CVB3c$_{(s)}$ binding to cells was calculated as

$$100\% - \left[\frac{\text{fraction of added [35S]}-\text{CVB3c}_{(s)}\text{ that binds to treated cells}}{\text{fraction of added [35S]}-\text{CVB3c}_{(s)}\text{ binding to untreated (control) cells}} \times 100\right].$$

MAb inhibition of CVB3c$_{(s)}$ infection of HeLa cells

We then examined whether anti-DAF MAbs inhibit cardiovirulent CVB3 virus from infecting susceptible cells (Fig. 2). Anti-DAF MAb 914 strongly inhibited plaque formation by the CVB3-CG, CVB3-CGP1V, CVB3-SH, CVB3-∅, and CVB3-VR30 strains and moderately inhibited plaque formation in HeLa cells by the CVB3-N, CVB3-NR, and CVB3-20 strains. In contrast, anti-DAF MAb IA10 was generally less effective at inhibiting CVB3c$_{(s)}$ plaque formation in HeLa cells, when used at the same concentration as MAb 914. Control MAb 653 (anti-CD4) did not affect CVB3c$_{(s)}$ plaque formation in HeLa cells (percentage of inhibition of plaques, 0%; results not shown).

[^{35}S]CVB3c$_{(s)}$ binding to PI-PLC-treated HeLa cells

Since MAbs against DAF could theoretically cause steric hindrance of the binding of virus to other cell surface molecules, we also examined CVB3c$_{(s)}$ binding to DAF-depleted HeLa cells. To do this, cell were pretreated with phosphatidylinositol phospholipase C (PI-PLC), an enzyme which cleaves GPI-anchored molecules such as DAF from the cell surface (Davitz *et al.,* 1986; Medof *et al.,* 1986). DAF depletion of HeLa cells by PI-PLC enzyme was confirmed by flow cytometry analysis (Fig. 3). Following PI-PLC treatment, the intensity of DAF staining decreased (left shift in the peak) by approximately 75%. A reduction in binding of the ^{35}S-labeled CVB3-CG, CVB3-SH, CVB3-∅, and CVB3-CGP1V strains to the PI-PLC-treated HeLa cells was also observed (Fig. 4). In contrast, PI-PLC treatment of HeLa cells reduced binding of ^{35}S-labeled CVB3-NR and CVB3-N strains by only 30% and the binding of [^{35}S]CVB3-VR30 and CVB3-20 strains by less than 20% (Fig. 4).

Effect of virus infection on DAF expression on HeLa cells

The effect of CVB3c$_{(s)}$ on DAF expression at the cell surface was also monitored. Infection of HeLa cells by all CVB3c$_{(s)}$ for 3 h led to a reduced expression of DAF on the cell surface, by 10–25%, compared to uninfected control cells (Fig. 5). Binding of virus most likely internalizes the DAF receptor, since eclipse of bound CVB3 in HeLa cells generally requires only several minutes at 37°C (Zajac and Crowell, 1969).

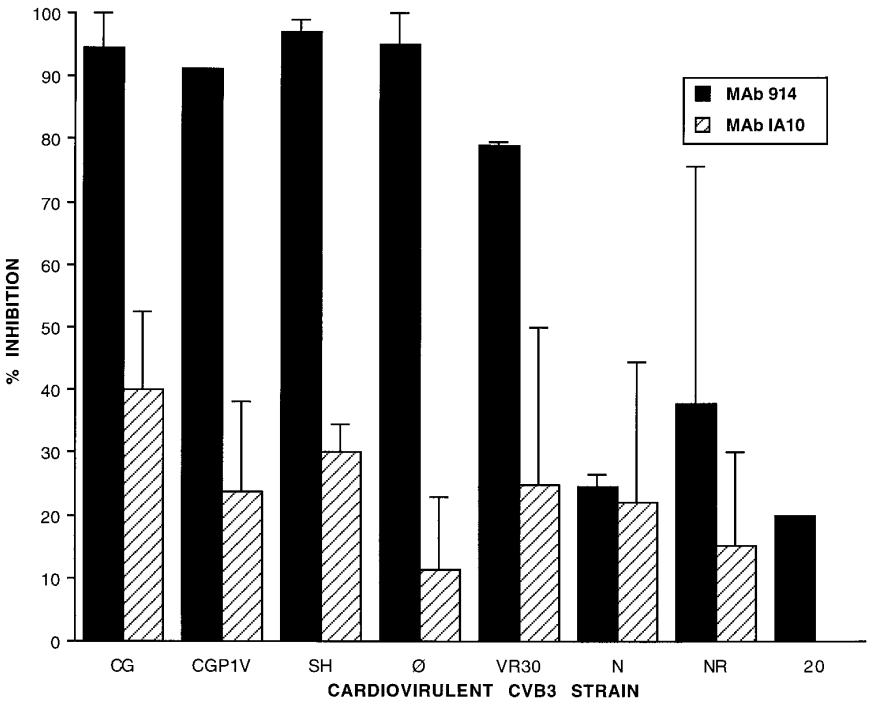

FIG. 2. Inhibition of CVB3c$_{(s)}$ plaque formation on HeLa cells pretreated with anti-DAF MAbs 914 or IA10. HeLa cell monolayers plated in 6-well Costar dishes at a density of 1×10^6 cells/well were preincubated with 1 ml media containing 50 μg/ml of MAb IA10 or 914, or 1 ml of media only, at 37°C for 1 h. After washing, 1 ml containing 50 PFU/ml of each virus was added at 37°C for 1 h. The monolayers were washed, plaque overlays were added for 3 days, and then plaques were enumerated. The CVB3-20 strain plaques inefficiently on HeLa cells, so infectivity for this strain was monitored by a $TCID_{50}$ assay. All experiments were performed in triplicate. Inhibition of CVB3c$_{(s)}$ infection of HeLa cells was determined as

$$100\% - \left[\frac{\text{No. CVB3c(s) PFU on MAb}-\text{treated cells}}{\text{No. CVB3c(s) PFU on untreated cells}} \times 100\right].$$

Complement studies

We examined the ability of CVB to interact with the DAF-related complement molecules CD46, CD35, Factor H, and C4bp. The cardiovirulent CVB3-CG strain did not interact directly with any of these molecules (percentage of inhibition, 0; results not shown). There was no reduction in virus infection on HeLa monolayers pretreated with anti-CD46 MAb. Moreover, there was no detectable binding of [^{35}S]CVB3-CG to human blood cells. Finally, when virus was pretreated with Factor H or C4bp, there was no reduction in infection in HeLa cell plaque assays compared to untreated virus controls.

Ad2-CVB competition binding assays

To examine the competitive binding interactions between Ad2 and CVB, competition assays were performed. Pretreatment of HeLa cells with unlabeled Ad2 virus strongly inhibited radiolabeled [^{35}S]Ad2 binding to the cells (Fig. 6). [^{35}S]Ad2 binding to cells was also inhibited in cells pretreated with CVB serotypes 1–6 (Fig. 6). Virus binding to HeLa cells was most strongly inhibited by pretreatment with odd serotypes CVB 1, 3 and 5, whereas even-CVB serotypes 2, 4, and 6 were less competitive. [^{35}S]Ad2 binding to HeLa cells was also reduced in cells pretreated with CVB3c$_{(s)}$ (Fig. 7). Virus binding was strongly inhibited by strains CVB3-CG, CVB3-∅, and CVB3-VR30 and moderately inhibited by pretreatment with CVB3-CGP1V and CVB3-SH, but was not inhibited by strains CVB3-N, CVB3-NR, and CVB3-20.

Ad2 binding to DAF

We also monitored the ability of Ad2 to bind to DAF by examining Ad2 binding to HeLa cells pretreated with anti-DAF MAbs or Ad2 binding to DAF-reduced (PI-PLC-pretreated) HeLa cells. [^{35}S]Ad2 binding to HeLa cells was moderately inhibited by pretreatment of HeLa cells with anti-DAF MAb IA10 (Fig. 8). In contrast, virus binding was not inhibited by anti-DAF MAb 914 (Fig. 8). [^{35}S]Ad2 binding to HeLa cells rendered DAF-deficient by treatment with the enzyme PI-PLC was significantly increased, compared to virus binding to untreated HeLa cells (Fig. 8).

DISCUSSION

CVB3 is implicated in the pathogenesis of myocarditis and dilated cardiomyopathy (Liu *et al.,* 1996; Martino *et al.,* 1994, 1995b,c; Woodruff, 1980). Many CVB3 strains can infect and replicate in the heart, as demonstrated in

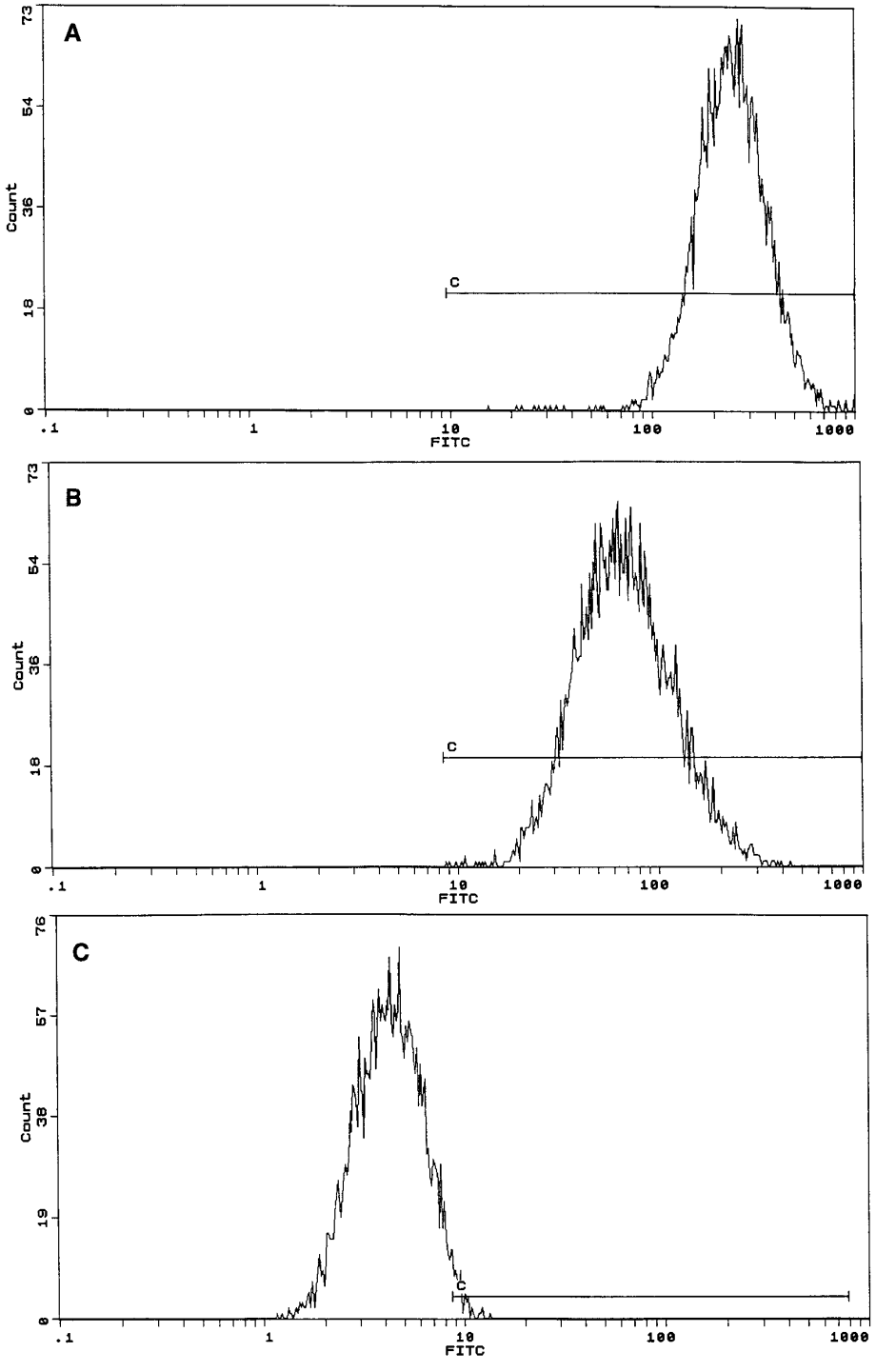

FIG. 3. Characterization of DAF-depleted HeLa cells by flow cytometry analysis. Versene-dispersed HeLa cells aliquoted at 5×10^6 cells/test were treated with 0.2 ml RPMI containing 0.5 units of phosphatidylinositol phospholipase C (PI-PLC, from *Bacillus cereus;* Boehringer Mannheim, Laval Quebec, Canada) at 37°C for 1 h. Cells were washed three times and labeled with 10 μl of undiluted FITC-conjugated anti-DAF MAb 737F on ice for 30 min. The cells were washed and resuspended in 1 ml PBS, and DAF expression/loss was monitored by flow cytometry. (A) Untreated HeLa cells. (B) HeLa cells treated with PI-PLC. (C) HeLa cells labeled with control MAb (FITC-conjugated rabbit (Fab')2 anti-rat IgG; Serotec).

murine model systems. However, only some strains are found to be cardiovirulent, because only these strains initiate disease processes which lead to inflammation of the myocardium and severe destruction of myocardial tissue. The genesis of viral heart disease likely represents a complex interplay of both virus-encoded and host-derived factors. In this study we examined one of these factors: the capacity of cardiovirulent CVB3 strains to interact with the DAF cell surface receptor.

The characteristics of the CVB3 strains used in this study are summarized in Table 1 Three lines of evidence show that the cardiovirulent CVB3 strains used in this

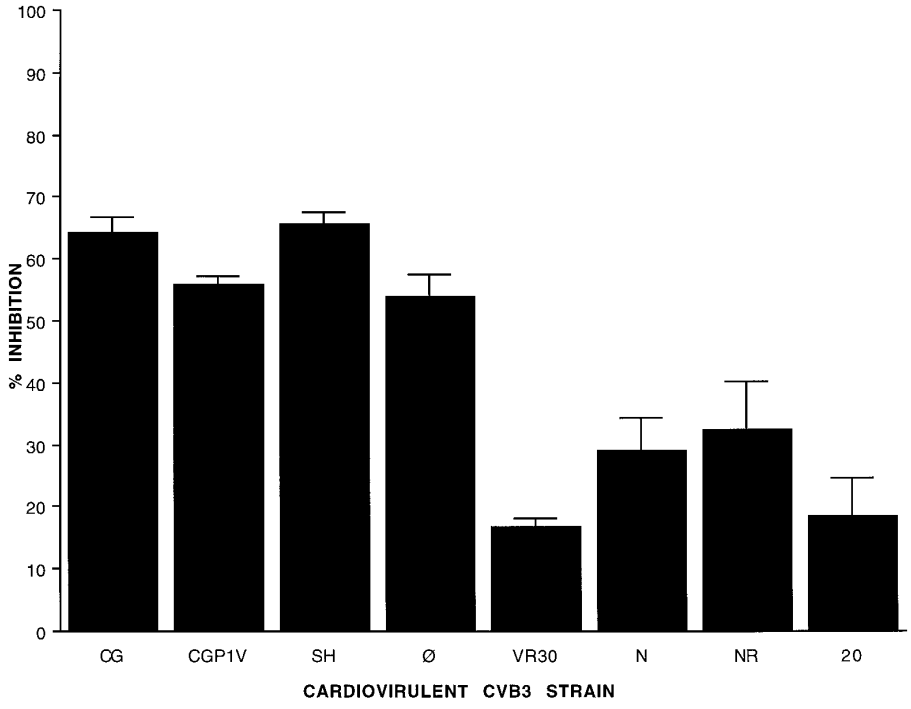

FIG. 4. Reduction in [^{35}S]CVB3c$_{(s)}$ binding to PI-PLC-treated DAF-depleted cells. PI-PLC-treated HeLa cells (unlabeled) were prepared as described in Fig. 3. They were used in binding assays as described in Fig. 1 The experiments were performed in quadruplicate for each virus strain.

study interacted with DAF on the cell surface. (1) Binding of radiolabeled [^{35}S]CVB3c$_{(s)}$ to HeLa cells decreased when cells were preincubated with anti-DAF MAbs, compared to control MAbs or media. (2) Plaque formation by CVB3c$_{(s)}$ was reduced when HeLa cell monolayers were preincubated with anti-DAF MAbs, showing that blockage of DAF reduced CVB3c$_{(s)}$ infection. (3) Binding of CVB3c$_{(s)}$ to DAF-depleted PI-PLC-treated HeLa cells was reduced, compared to untreated HeLa cell controls.

The present study showed that although all the strains tested showed interactions with DAF, some CVB3c$_{(s)}$ interacted more strongly with DAF than others. DAF interactions were most pronounced for the viruses which produce severe disease in the murine model (CVB3-CG, CVB3-SH), compared to those which produce less severe disease (CVB3-20, CVB3-NR) *in vivo.*

Interestingly, the CVB3-∅ strain interacted significantly more strongly with DAF than the parental CVB3-20 strain. There are several mutations in the capsid region of CVB3-∅ relative to CVB3-20 (Chapman *et al.,* 1994; Tracy *et al.,* 1992; Tu *et al.,* 1995), and it is possible that one or more of these mutations could result in a significantly stronger DAF-binding phenotype.

The CVB3-N strain exhibited a weaker DAF-binding phenotype, similar to the interactions observed for CVB3-NR. Interestingly, nucleotide sequencing and phylogenetic analysis of the (receptor-binding) capsid region of the strains indicated that CVB3-NR and CVB3-N are most closely related to each other, as compared to the other CVB3c$_{(s)}$ (Martino *et al.,* 1997), consistent with the similar binding patterns observed for these two viruses.

The CVB3-VR30 strain is presumably noncardiovirulent, and its interactions with DAF were distinct from those noted for the cardiovirulent strains of virus. Anti-DAF MAbs strongly blocked virus binding (Fig. 1) and infection (Fig. 2) of HeLa cells. However, CVB3-VR30 binding was only weakly affected by PI-PLC digestion of cells (Fig. 4). Although the reason for this discrepancy is unclear, it may indicate that of all the strains tested, the CVB3-VR30 strain most requires an additional receptor(s) other than DAF for efficient binding to the cell surface. This is consistent with previous reports that MAbs can block CVB3-VR30 binding to the cell surface, but that this strain binds to DAF only weakly (Shafren *et al.,* 1995) or not at all (Bergelson *et al.,* 1995).

The extent to which anti-DAF MAbs interfered with binding/infection of HeLa cells by a CVB3c$_{(s)}$ depended on the epitope to which the antibody was directed. MAb 914 (clone BRIC 216) binds to the DAF molecule at SCR3 (Coyne *et al.,* 1992) and was most effective at reducing CVB3c$_{(s)}$ infection of HeLa cells. In preliminary studies, MAb 737 (clone BRIC 110), which binds to SCR2 (Coyne *et al.,* 1992), produced similar results when tested with the CVB3-CG strain (unpublished observations). In contrast, MAb IA10, which binds DAF at SCR1 (Coyne *et al.,* 1992), does not block CVB3 efficiently. These findings are consistent with previous reports that anti-DAF MAbs recognizing SCR2 and SCR3 are most effective at blocking virus attachment and internalization and that a CVB-binding site is likely located within or near these domains (Bergelson *et al.,* 1995; Shafren *et al.,* 1995).

Complement regulatory molecules which are structur-

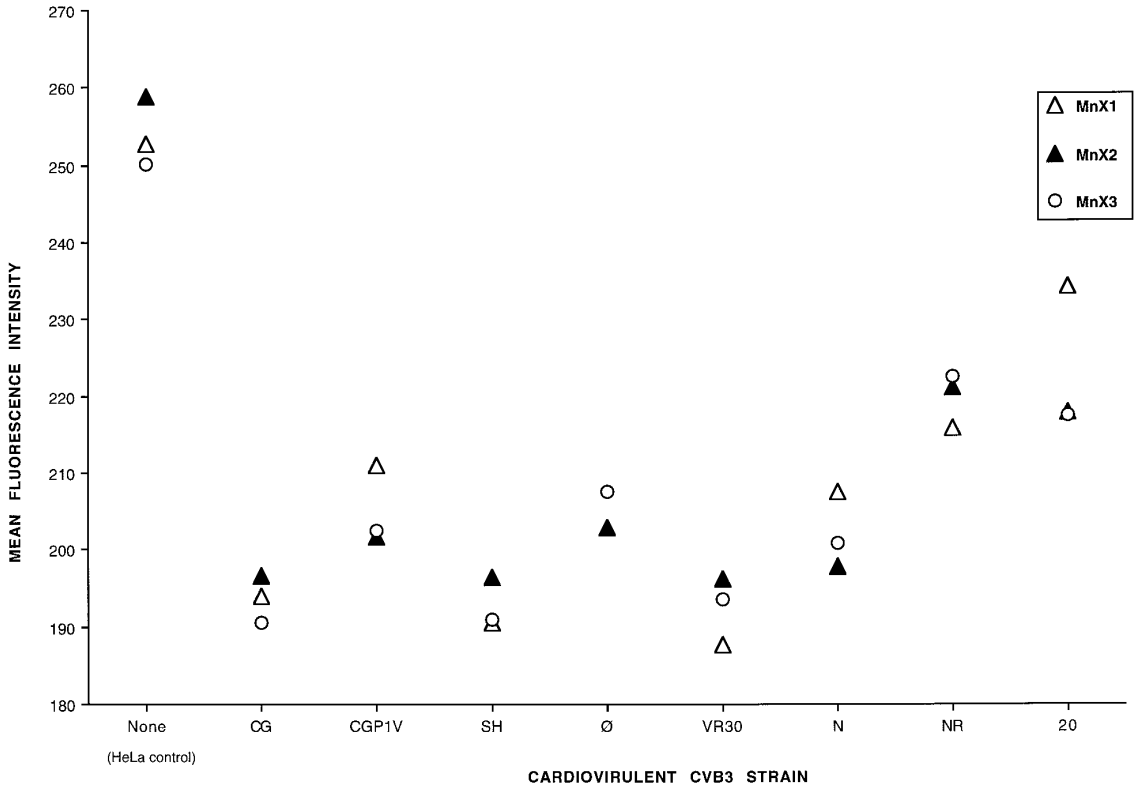

FIG. 5. DAF reduction on the surface of CVB3c$_{(s)}$-infected cells. Versene-dispersed HeLa cells aliquoted at 1×10^6/test were resuspended in 200 μl RPMI containing 1×10^8 PFU of virus, for 1 h at 25°C. Cells were washed and further incubated in complete media for 2 h at 37°C. Cells were labeled with FITC-conjugated anti-DAF MAb 737F as described in Fig. 3, and DAF expression/loss was monitored by flow cytometry. MnX refers to the mean fluorescence intensity of the sample under analysis. The assay was performed in triplicate, and each symbol represents one value obtained.

ally and/or functionally related to DAF, including CR1 (CD35), MCP (CD46), C4-binding protein, and Factor H (Hourcade *et al.,* 1989), do not interact with the cardiovirulent CVB3-CG strain, indicating that the site of virus binding is unique to the DAF molecule. The possibility exists that other strains not tested could interact with DAF-related ligands.

Cardiovirulent CVB3 strains bind to DAF. In light of this observation, two important questions arise. First, DAF is not a receptor in the classical sense, because it alone does not promote lytic infection of cells (Bergelson *et al.,* 1995; Shafren *et al.,* 1995). How then, does CVB3c$_{(s)}$ binding to DAF play a role in the pathogenesis of CVB-induced heart disease? Second, are all DAF-binding viruses cardiovirulent?

In addressing the first question, there are several possible mechanisms by which DAF binding could play a role in the pathogenesis of viral heart disease. (1) DAF binding may facilitate or promote virus presentation to an additional cell surface receptor(s), thus enhancing virus virulence. (2) DAF distribution may promote viral tropism for specific organ sites. (3) Binding of CVB3c$_{(s)}$ to DAF could play a role in the initiation of signaling cascades, with pathogenic consequences. For example, DAF is a glycolipid-anchored protein which associates with the T-cell-activating tyrosine kinases p56^{lck} and p59^{fyn} (Davis *et al.,* 1988; Shenoy-Scaria *et al.,* 1992). T-cell activation, which is a hallmark feature of myocarditis, does not occur in transgenic mice lacking the signaling molecule p56^{lck}. These mice are resistant to CVB heart disease (Martino *et al.,* 1995a). (4) Loss of DAF expression on CVB-infected cells, as was noted in this study, may make the cells more susceptible to complement-mediated lysis. This could facilitate release of progeny virus from some cell types, further enhancing virus virulence. (5) Finally, it is interesting to note that murine DAF (Spicer *et al.,* 1995) can be induced and regulated by estrogen (Song *et al.,* 1996). It has previously been reported that estrogen is protective in murine models of CVB-induced heart disease (Huber *et al.,* 1982). Moreover, in both mice and humans, virus-induced heart disease is more severe and more prevalent in males than in females (Martino *et al.,* 1995b).

In response to the second question, this study focused on CVB3 variants which are known to cause heart disease in well-defined model systems. All the cardiovirulent strains of CVB3 tested here bound to DAF. However, this does not presume that all DAF-binding viruses are cardiovirulent. Indeed, prototype strains of CVB1, CVB5, CVA21, and several echoviruses bind to DAF (Bergelson *et al.,* 1994, 1995; Shafren *et al.,* 1995, 1997b). But additional characteristics of each virus strain, including binding to other receptors, internalization, replication, and progeny production, come into play in determining dis-

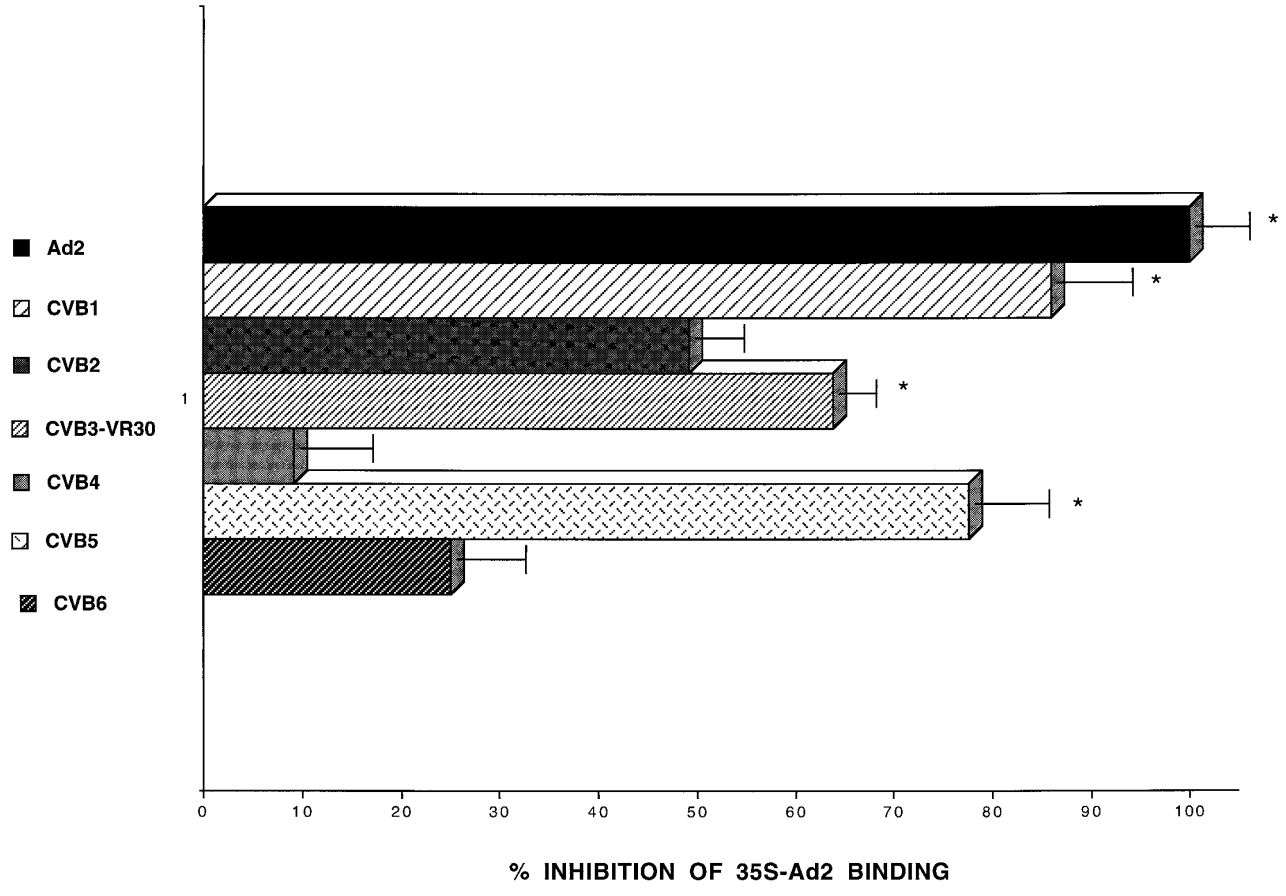

FIG. 6. Adenovirus 2 and CVB serotypes 1–6 competitive binding assays. Versene-dispersed HeLa cells aliquoted at 5×10^6 cells/test were preincubated in 0.2 ml of media containing 1×10^8 PFU of unlabeled virus strain (Ad2 or CVB1-6) or 0.2 ml of media, for 1 h at 37°C. Cells were washed and further incubated with 5×10^4 cpm of [^{35}S]Ad-2 virus, for 1.5 h at 37°C. [^{35}S]Ad2 binding was monitored by scintillation spectroscopy, as described in Fig. 1. The experiments were performed at least three times for each virus serotype. The asterisk denotes statistical significance ($P < 0.001$).

ease outcome. Nevertheless, future studies examining the cardiopathogenicity of other enteroviruses would be interesting, particularly in light of the observation that strains including CVB1, CVB5, CVA, and echoviruses have been associated with heart disease in humans (WHO/ISFC Task force, 1980).

In this study, we also examined the ability of Ad2 to bind to DAF, as well as the competitive binding characteristics between Ad2 and CVB serotypes 1–6 and CVB3c$_{(s)}$. These studies stem from the original reports that CVB and Ad2 fiber can compete for a receptor (Lonberg-Holm *et al.,* 1976). We found that the odd-numbered CVB 1, 3, and 5 serotypes competed most strongly with Ad2 for binding to the HeLa cell surface, compared to the even-numbered CVB 2, 4, and 6 serotypes (Fig. 6). One possible explanation for this observation is that the odd-numbered CVB serotypes exhibit greater binding affinities than the even-numbered ones (Crowell, 1976) and thus more rapidly saturate available cell surface receptors. Additionally, it has been noted that CVB 1, 3, and 5 can bind to DAF, whereas CVB 2, 4, and 6 do not bind DAF (Bergelson *et al.,* 1995; Shafren *et al.,* 1995), and the ability to saturate the DAF receptor could be important in competing with Ad2 for cell surface binding. In support of this latter hypothesis, we have also observed that cardiovirulent CVB3c$_{(s)}$ strains which interacted most strongly with DAF were more effective at inhibiting Ad2 binding to HeLa cells than cardiovirulent strains which showed weaker interactions with DAF (Fig. 7).

It seems unlikely that Ad2 binds directly to DAF. Anti-DAF MAb 914, which binds to the third domain (SCR3) near the site of CVB attachment, does not affect Ad2 binding to the cell surface. Anti-DAF MAb IA10, which binds to the outermost domain (SCR1), does block approximately 50% of Ad2 binding, but this is likely due to steric hindrance. The observation that DAF-reduced HeLa cells are significantly more capable of binding Ad2 than normal HeLa cells also points to the notion that pretreatment with DAF-binding viruses or antibodies sterically blocks the ability of Ad2 to bind to its natural receptors. It also indicates that DAF- and Ad2-binding receptors (such as coxsackie adenovirus receptor (CAR) (Bergelson *et al.,* 1997a; Carson *et al.,* 1997; Tomko *et al.,* 1997) or the Ad2-binding integrins $\alpha_v\beta_3$ or $\alpha_v\beta_5$ (Wickham *et al.,* 1993), or Ad2 fiber-binding human fibronectin type

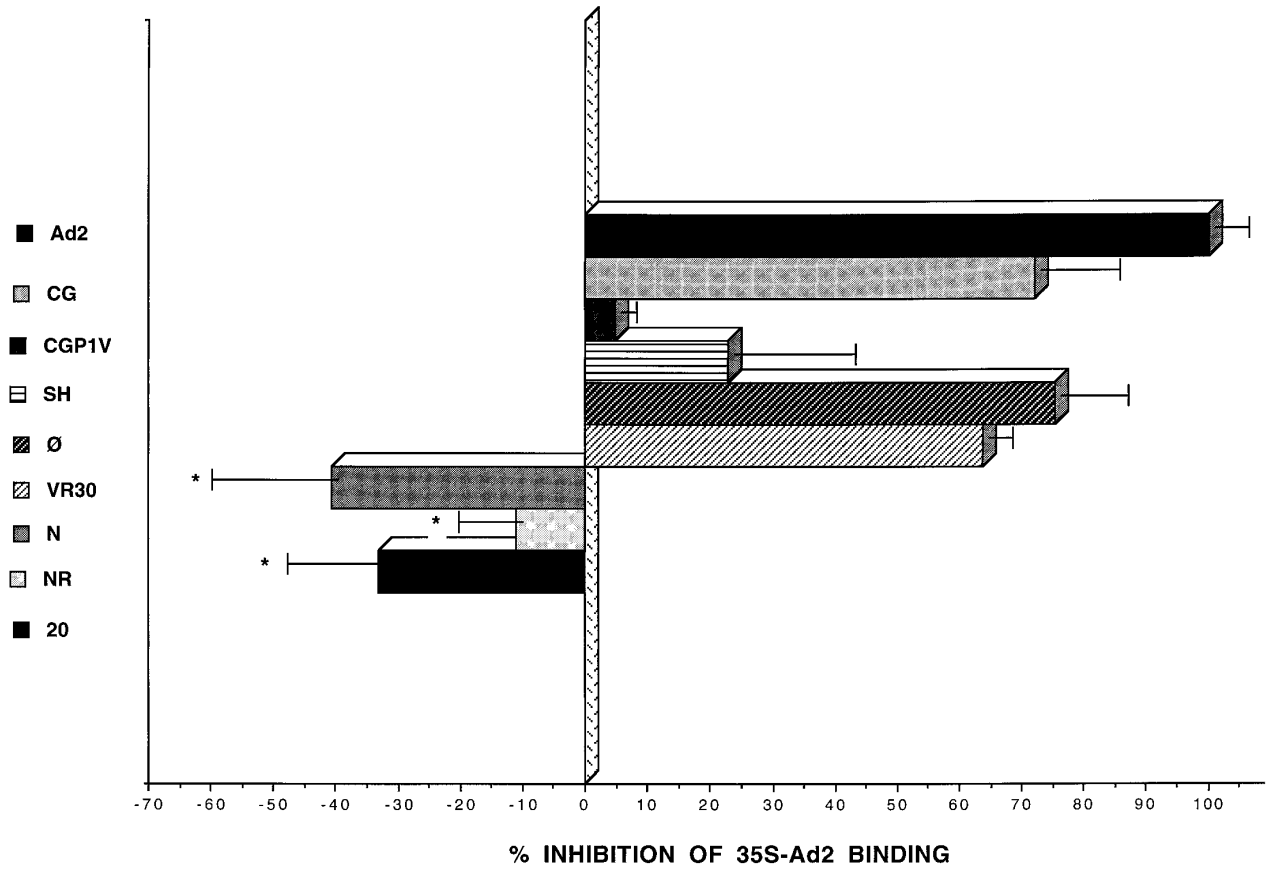

FIG. 7. Adenovirus 2 and CVB3c$_{(s)}$ competitive binding assays. Assays were performed as described in Fig. 6, but used CVB3c$_{(s)}$ instead of CVB1–6. The experiments were performed in triplicate for each virus strain. The asterisk denotes statistical significance ($P < 0.001$).

III (Hong and Boulanger, 1995), or the MHC class I α2 domain (Hong *et al.,* 1997)) probably colocalize on the HeLa cell membrane. Indeed, it has previously been reported that DAF also colocalizes with ICAM-1 (Shafren *et al.,* 1997b), the receptor shared by coxsackievirus A21 (Shafren *et al.,* 1997a) and the major group rhinoviruses (Greve *et al.,* 1989; Stauton *et al.,* 1989; Tomassini *et al.,* 1989).

In this study, cardiovirulent strains of CVB3 interacted with the DAF receptor, but Ad2 virus did not, shedding some light on the different tissue tropisms, disease manifestations, and host ranges noted for each virus. It is likely that CVB binding to DAF is only one of many virus-determined factors involved in viral heart disease. Additional mechanisms by which CVB targets heart tissue and initiates aberrant immune responses will undoubtedly become clearer once reagents to recently identified CVB receptor molecules like CAR (Bergelson *et al.,* 1997a; Carson *et al.,* 1997; Tomko *et al.,* 1997) and a 100-kDa nucleolin-like protein (Raab de Verdugo *et al.,* 1995) become available. Understanding these interactions is important for an understanding of the disease processes that occur and perhaps will open new avenues for the treatment of CVB heart disease.

MATERIALS AND METHODS

Cell lines

HeLa (ATCC CCL-2) and Vero (ATCC CCL-81) cells were grown in RPMI 1640 media supplemented with $NaHCO_3$ to pH 7.5, 0.5% penicillin and streptomycin, and 10% fetal calf serum (FCS). All media and supplements were from Gibco/BRL Life Technologies Canada, Burlington, Ontario, Canada.

Viruses

The CVB3 cardiovirulent strains (CVB3c$_{(s)}$) -SH, -NR, -N, -CG, -20, and -∅ were from the laboratories of Charles Gauntt and Larry Chow. The CVB3-VR30 strain was obtained from the American Type Culture Collection. The passage strain CGP1V refers to the CVB3-CG strain passaged once through Vero cells. The characteristics of these strains are summarized in Table 1. Adenovirus type 2 was obtained from the American Type Culture Collection. Stocks of these viruses were prepared by passaging them through HeLa cell cultures (with Vero cells used for CGP1V). Stocks were freeze–thawed 3×, clarified by centrifugation, titered by plaque assay on HeLa cells, aliquoted, and stored at −70°C.

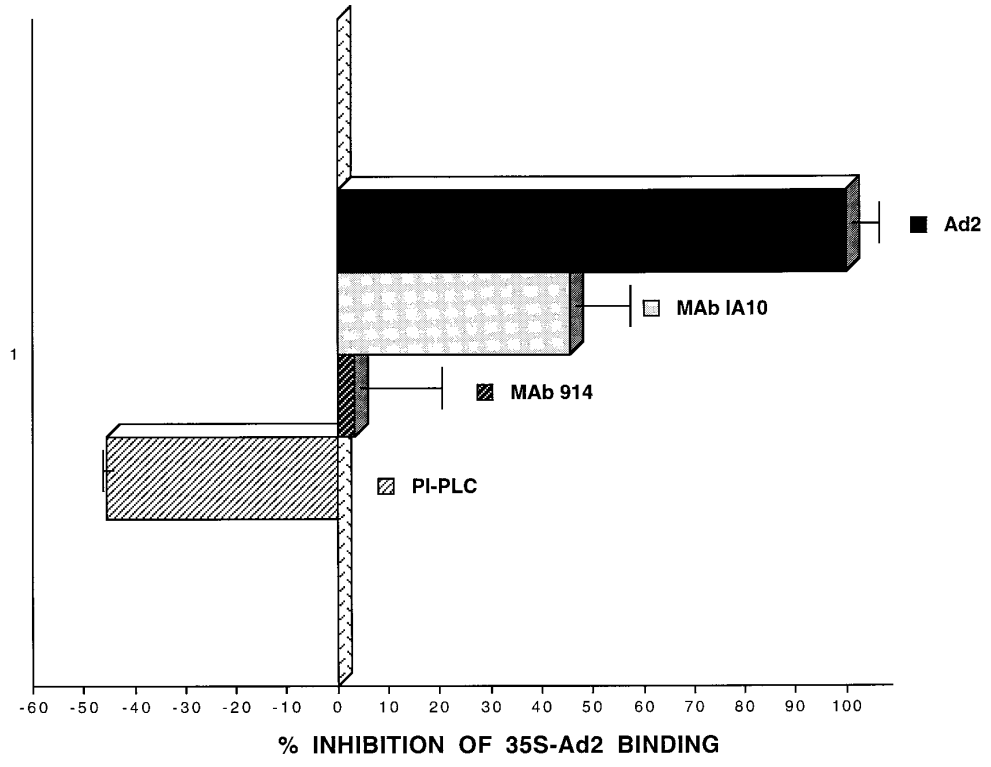

FIG. 8. Inhibition of [^{35}S]Ad2 binding to HeLa cells. HeLa cells pretreated with anti-DAF MAbs 914 and IA10 were prepared as described in Fig. 1. PI-PLC-treated cells were prepared as described in Figs. 3 and 4. Pretreated cells were then incubated in 200 μl RPMI containing 5×10^4 cpm of [^{35}S]Ad2, for 15 h at 37°C. Virus binding was determined as described above. The experiments were performed at least three times for each assay.

Virus radiolabeling and purification

HeLa cell monolayers in 10-cm^2 culture dishes were incubated with the infecting CVB virus (10 PFU/cell) for 30 min at 37°C. Monolayers were washed and then incubated at 37°C in media deficient in methionine for 2.75 h. To each dish, 6 ml of media containing 100 μCi of [^{35}S]methionine (Amersham Life Science, Oakville, Ontario, Canada) was added for an additional 3.75 h. The infected cell monolayers were washed, collected by scraping into 2 ml of collection buffer (15% sucrose, 0.01 M NaCl, 0.01 M Tris–HCl, 0.05 M $MgCl_2$), and subjected to three freeze–thaw cycles to release the virus. The lysate was centrifuged at 12,000 *g* for 15 min to remove the cell debris. The supernatant was overlaid on sucrose gradients [15–45% (wt/wt) in collection buffer] and centrifuged at 24,000 rpm at 25°C for 3 h in an SW28 rotor (Beckman, Mississauga, Ontario, Canada). Fractions with peak radioactivity and viral titer were pooled and dialyzed in 10K Slide-A-Lyzer Cassettes (Pierce, Rockford, IL) against RPMI medium. Virus titer was determined by plaque assay and aliquots were stored at −70°C.

For adenovirus type 2, HeLa cell monolayers were incubated at an m.o.i. of 5 PFU/cell for 1 h at 37°C, washed, and then incubated in regular media. After 16 h, the medium was changed to 6 ml of methionine-deficient RPMI which contained 100 μCi of [^{35}S]methionine, and cell monolayers were further incubated for 6 h. Finally, the medium was changed to 10 ml containing (1:1) regular RPMI:methionine-deficient RPMI supplemented with 300 μCi of [^{35}S]methionine, and cells were incubated for 48 h. The cells were collected by scraping into 2 ml of collection buffer, then subjected to five freeze–thaw cycles to release virus, and the lysate was centrifuged at 12,000 *g* for 15 min to remove cell debris. The supernatant was overlaid on sucrose gradients [15–60% (wt/wt) in collection buffer] and centrifuged as described above. Virus containing fractions were pooled, dialyzed, titred, and stored as described above.

Plaque assays

HeLa cells plated in 6-well Costar dishes at a density of 1×10^6 cells/well were incubated with serial 10-fold dilutions of CVB3c$_{(s)}$ in 0.2 ml of RPMI medium (without FCS), for 1 h at 37°C. Unbound virus in the inoculum was removed and an overlay of 2× RPMI medium supplemented with 20% FCS and 1.4% agarose [1:1 (vol/vol)] was added. The monolayers were incubated for 3 days, fixed in 2% Formalin, and stained with 0.1% crystal violet dye. Adenovirus type 2 was titered in the same manner, except that the monolayers were incubated for 10 days to allow for plaque formation.

$TCID_{50}$ assays

HeLa cells plated in 96-well Costar dishes at a density of 6.25×10^4 cells/well were incubated with serial two-

fold dilutions of $CVB3c_{(s)}$ in 0.02 ml of RPMI medium (without FCS), for 1 h at 37°C. Unbound virus in the inoculum was removed, and RPMI medium supplemented with 10% FCS was added. The monolayers were incubated for 3 days, fixed in 2% Formalin, and stained with 0.1% crystal violet dye. The end point was taken as the last dilution in which 50% CPE was noted.

MAbs and complement proteins

Anti-DAF MAb 1A10 was obtained from Cedarlane Laboratories Ltd., Hornby, Ontario, Canada. Anti-DAF MAb 914 (clone BRIC 216) and anti-DAF MAbs 737 and 737F (FITC conjugated) (clone BRIC 110) and MAb 695 to membrane cofactor protein (anti-MCP/CD46; clone J4-48), MAb 554 (anti-CR1/CD35; clone E11), and MAb 653 (anti-CD4; clone B-B14) were obtained from Serotec Canada, Toronto, Ontario, Canada. Complement Factor H and C4-binding protein were purchased from Sigma Immunochemicals.

Anti-DAF MAb inhibition of $[^{35}S]CVB3c_{(s)}$ binding to HeLa cells

Versene-dispersed HeLa cells were preincubated with anti-DAF MAb 914 (anti-CD4 MAb 653 was used as control). Radiolabeled $[^{35}S]CVB3c_{(s)}$ was then added to the MAb-treated cells. Supernatant fluid and two subsequent washes were collected, as was the cell pellet. Unbound and cell bound virus were determined by monitoring the ^{35}S cpm in supernatants and cell pellets, respectively, by scintillation spectroscopy.

Anti-DAF MAb inhibition of $CVB3c_{(s)}$ infection of HeLa cells

HeLa cells plated on Costar dishes were preincubated with anti-DAF MAb 914 or MAb IA10 (or anti-CD4 MAb 653 as control) and then with 50 PFU/ml of each virus. Infection was monitored by plaque assay for all strains except CVB3-20, which was monitored by a $TCID_{50}$ assay.

Effects of DAF depletion on binding of $CVB3c_{(s)}$

Versene-dispersed HeLa cells were pretreated with PI-PLC (from *Bacillus cereus;* Boehringer Mannheim, Laval Quebec, Canada). Cells were then labeled with FITC-conjugated anti-DAF MAb 737F as described above. DAF expression/loss was monitored by flow cytometry. DAF-deficient HeLa cells were then used in binding assays with $[^{35}S]CVB3c_{(s)}$ by methods described above.

$CVB3c_{(s)}$ binding and DAF expression

Versene-dispersed HeLa cells were preincubated with each CVB3 strain. The cells were washed and then further incubated in RPMI medium. Cells were labeled with FITC-conjugated anti-DAF MAb 737F and processed through flow cytometry, as described above.

Complement studies

Versene-dispersed HeLa cells were preincubated with anti CD46-MAb 695, by the same methods described for anti-DAF MAbs. After washing, radiolabeled $[^{35}S]$CVB3-CG was added to the MAb-treated cells. Virus binding was monitored by scintillation spectroscopy, as described above. CD35-binding assays were performed using human blood cells, since the CD35 molecule is not expressed on HeLa cells (unpublished observations). Whole human blood (1 ml) was collected on ice, spun down at 1000 *g* for 5 min to remove plasma, washed in RPMI, and then aliquoted at 5×10^6 cells/test. Binding assays with $[^{35}S]$CVB3-CG were performed as described above. Factor H or C4bp was preincubated with 50 PFU/ml of CVB3-CG for 1 h at 37°C and then virus infection was monitored by plaque assay as described above.

Ad2-CVB competition binding assays

Versene-dispersed HeLa cells were preincubated with unlabeled CVB virus or unlabeled Ad2 for 1 h at 37°C. The cells were washed and further incubated with radiolabeled $[^{35}S]$Ad2 for 15 h at 37°C. $[^{35}S]$Ad2 binding was monitored by scintillation spectroscopy, as described above.

Ad2 binding to DAF

Versene-dispersed HeLa cells were preincubated with anti-DAF MAb 914 or anti-DAF MAb IA10 or PI-PLC enzyme, as described above. The cells were washed, and radiolabeled $[^{35}S]$Ad2 was then added. $[^{35}S]$Ad2 binding was monitored by scintillation spectroscopy, as described above.

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